Histochemical Studies of PAS Positive Reticular Cells in Mouse Thymus, with Special Reference to Their Relation to Mast Cells and Pancreatic B-Cells.

The occurrence of PAS positive cells of reticular type was described by METCALF and ISHIDATE (1961), ITO and HOSHINO (1962). These cells were reported to be sudanophilic by BOMPIANI (1914), LOEWENTHAL and SMITH (1952), SPICER (1960), ITO and HOSHINO (1962). In the course of irradiation experiments of mice, the author observed cells with aldehyde-fuchsin and PAS positive granules, which seemed to correspond to the cells described by authors mentioned above. Although these cells have been named variously, for example, as “Infiltrationszellen” by FULCI (1913) and as “PAS cells” by METCALF (1961), the name of the last author will be used in this report.

The PAS cells resemble to mast cells in shape, sudanophilia, and the affinity to aldehyde-fuchsin. Though the PAS cells are generally much larger than mast cells, there may occur smaller ones which are not distinguishable from ordinary mast cells on the basis of their size. Moreover, the appearance of mast cells in the thymus has been observed by many authors (cf. CSABA 1965 and BURNET 1965). Thus, it was one of the objectives of the present work to investigate the relations, if any, of the both kinds of cell. Special attention was paid as to whether the PAS cells are concerned with the mastocytogenesis, and whether any transformation from PAS cells to mast cells, or vice versa, may occur or not.

Next comes the question about the relation between PAS cells and pancreatic B-cells. PANSKY and his co-workers who treated of a special type of reticular cells with the affinity to aldehyde-fuchsin in AKR mice thymus, postulate that these cells secrete or store an insulin-like material (PANPSKY and HCUSE 1965, PANPSKY et al. 1965). As these reticulum cells seem to be identical with our PAS cells, it became of keen interest to examine the relation between the PAS cells and the islet B-cells. In the present work, the histochemical properties of the three types of cell, i.e., mast cells, PAS cells, and B-cells of the pancreas, were compared in an attempt to find out some characteristics of the PAS cells.

I. Materials and Methods

Histochmmical studies: Animals used were 60 Db mice of either sex, aged 2 months, 3
months, 6 months, and 1 year, in addition 2 AKR mice of either sex, aged 3 months. Ten Db and 2 AKR mice were sacrificed by decapitation, and 50 Db mice were killed 24 hours after total body X-irradiation of 850 r. Thymus, cervical lymphnodes, spleen, and pancreas were removed from each animal. Slices of these organs were fixed in (1) Bouin's fluid, (2) Carnoy's fluid, (3) absolute alcohol, (4) 4% basic lead acetate in 50% alcohol, and (5) 10% buffered formalin, embedded in paraffin, and sectioned serially at 4 μ. Some slices were fixed for 24 hours in 10% formol calcium (BAKER) and 10% buffered formalin, and sectioned on the freezing microtome at 12 μ. The following staining methods were applied to the sections: hematoxylin (CARACI) and eosin; aldehyde-fuchsin (original method by GOMORI and its 'condensed' modification by FUJITA and FUKUDA 1960) with and without preoxidation; periodic acid Schiff's reagent; performic acid Schiff's reagent; methyl-green pyronine in pH 4.8 acetate buffer; 0.1% toluidine blue in pH 2.5 and 4.5 acetate buffer; alcin blue; safranine with and without mild methylation of 4 hours at 37°C (SPICER 1960); 0.5% alcian blue with 0.4 M MgCl2 in pH 2.5 acetate buffer (SCOTT 1965); saturated Sudan black B in 60% alcohol for total lipid contents; 1% aqueous Nile blue solution for neutral lipids; the sulfuric acid test (SCHULZ) for cholesterol and cholesteryl esters; the acid hematein test (SMITH-DIETRICH) for phospholipids; the ferric-ferricyanide reaction (SCHMORL) for lipofuscins; the dithizone reaction (OKAMOTO) for zinc. Unstained sections were studied under the fluorescence microscope.

Zinc-dithizone reaction in vivo: According to the method of STAMPFL, 5 female Db mice, aged 8 months, were intravenously injected with dithizone in a dose of 120 mg per Kg body weight. Ten minutes after the injection animals were sacrificed by decapitation, and the thymus and pancreas were obtained from them. These materials were sectioned on the freezing microtome at 12 μ without any fixation, and examined immediately under a photomicroscope.

Extraction of zinc-dithizone compound: Five female Db mice, aged 8 months, were injected with dithizone, and the thymus, pancreas, lung, spleen and adipose tissue were removed from them according to the method mentioned above. Immediately, 700 mg of each material was sectioned on the freezing microtome with glass knives, and extracted in carbon tetrachloride at 4°C for 30 minutes. Then the extract was examined with Beckman-DU-spectrophotometer, and absorption curve was obtained for each sample.

The administration of alloxan: Five mice of either sex, aged 2 months and 8 months were injected intraperitoneally with alloxan in a dose of 200 mg per Kg body weight. Two, three and five hours after the injection the animals were killed, and each pancreas and thymus was taken from them. Materials were fixed in 10% formol and Bouin's fluid, embedded in paraffin, and sectioned serially at 4 μ. The sections were stained in dyes mentioned in histochemical studies.

II. Observations.

Histochemical studies.

In the thymus of normal and X-irradiated Db mice, aldehyde-fuchsin positive
cells, rounded, oval or reticular in shape, were seen scattered throughout the gland, but they were more abundant in the cortex, and had likewise the reactivity to the PAS reagent. These PAS cells increased in number with advancing age and after X-irradiation, although varying considerably in individual thymus. (Fig. 1)

The PAS cells were also recognized in the thymus of normal AKR mice which was larger in size than the thymus of Db mice of corresponding age; there was no significant difference in the frequency and histochemical properties of the PAS cells between the both strains.

Fig. 1. PAS cells in the thymus of an adult Db mouse. PAS reaction counterstained with hematoxylin. ×580

Fig. 2. Auto-fluorescence of PAS cells. Under the fluorescence microscope. ×580
The PAS cells were filled with somewhat coarse granules, larger than those of B-cells of the pancreas and smaller than those of mast cells. These granules appeared yellowish-white in unstained sections. A large, clear nucleus, ovoid or elongate in form, was recognized, generally suppressed on one side of the cytoplasm. Besides, in the majority of the cells, one or more pyknotic nuclei were seen among the granules, which probably correspond to the nuclei of phagocytized lymphoid cells as described by METCALF (1961), ITO (1962) and KLUG (1965).

Table 1. Reactivities of PAS cells, mast cells and pancreatic B-cells to some kinds of dyes.

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<th>PAS-cells</th>
<th>mast cells</th>
<th>B-cells</th>
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<tr>
<td>aldehyde-fuchsin</td>
<td>+</td>
<td>+</td>
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<tr>
<td>PAS</td>
<td>+</td>
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<td>PFAS</td>
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<td>pyronine</td>
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<td>toluidine blue</td>
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<td>alcian blue</td>
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<td>safranine</td>
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<td>Sudan black B</td>
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<td>Nile blue</td>
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<td>Shulz test</td>
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<td>Smith-Dietrich test</td>
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<td>Schmorl test</td>
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<td>Okamoto test</td>
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<td>auto-fluorescence</td>
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Histochemical properties of the three types of cell, i.e., mast cells, PAS cells, and pancreatic B-cells, were examined with several kinds of methods, the results of which are shown in Tab. 1.

Affinity to aldehyde-fuchsin was the only characteristic common to all the three cell types, though the stainability of PAS cells to aldehyde-fuchsin was weaker than in mast cells and B-cells of the pancreas (Fig. 3). PAS cells and mast cells were positive to Schiff's reagent after preoxidation with periodic acid and performic acid, colored intensively red with pyronine and showed sudanophilia. Though mast cells reacted metachromatically in toluidine blue and colored blue or violet with Nile blue, the PAS cells were green in both dyes. The alcian blue safranine reaction was positive only in the granules of mast cells, and Schmorl test was positive only in PAS cells.

Using embryonic mast cells of rats, COMBS (1965) reported a technic of alcian blue-safranine reaction which could distinguish between the granules with weakly sulfated and strongly sulfated mucopolysaccharides. By a shift from alcian blue, which stained the former granules, to safranine, which stained the latter, he identified four stages of granule maturation in mast cells. This method was applied to our sections, and no such cytoplasmic granules as seen in young mast cells were recognized in PAS cells.

OKAMOTO's method of detecting zinc in tissues with dithizone showed positive

Fig. 3. PAS cells in the thymus of an adult Db mouse. Aldehyde-fuchsin counterstained with Masson's trichrome method. ×830
results not only in the islets of Langerhans, but unexpectedly also in the PAS cells (Fig. 4). When exposed to ultraviolet light under the UV filter, PAS cells as well as mast cells emitted yellowish-brown auto-fluorescence of considerable intensity (Fig. 2). However, no such cells were observed in the lymphnodes and spleen.

**Zinc-dithizone reaction in vivo.**

According to the method of STAMPFL (1959), dithizone was injected intravenously in the Db mice and the sections were likewise examined by his method. In the sections of the pancreas, an intense zinc reaction was recognized in major portion of the islets, although it was not clear which cells of A- and B-types contained zinc. In the sections of the thymus, on the other hand, a considerable number of cells, rounded, oval or reticular in shape, revealed a faint purple-red color, and they were identified, by all their histochemical reactions, with the PAS cells.

**Extraction of zinc-dithizone compounds.**

As is well known, dithizone reacts with bismuth, copper, cadmium, lead and quicksilver as well as zinc. In 1959, STAMPFL examined the absorption maximum of various compounds of dithizone; for example, that of zinc-dithizone compound was described to be at 535 m\(\mu\) wave length and that of cadmium-dithizone compound at 520 m\(\mu\). Therefore we can find out which metal is concerned with a given compound of dithizone by the examination of its absorption maximum (Fig. 5).

![Fig. 4. PAS cells in the thymus of an adult Db mouse. Zinc-dithizone reaction with Okamoto method. \(\times830\)](image)

![Fig. 5. Absorption maximum of metal-dithizone compound. Solvent: carbon tetrachloride. (STAMPFL 1959)](image)

The absorption curve of the extract from the thymus showed the absorption maximum at 535 m\(\mu\) wave length, and it exactly coincided with that of zinc-dithizone compound. The curve obtained in the extract of the pancreas, showed, on the other hand, its maximum at about 520 (cadmium?) and 535 m\(\mu\) wave length (Fig. 6). The absorption curves of the lung and adipose tissues showed no distinct maximum, and that of spleen was nearly identical with zero-line.
The administration of alloxan.

In all the animals injected with alloxan, the sections of the pancreas showed more or less seriously damaged islets. The B-granules diminished markedly and the pericapillary spaces seemed to have enlarged. The nuclei of the B-cells fell into the state of serious pyknosis (Fig. 7). In contrast, none of these signs of cell degeneration was observed in the thymus (Fig. 8). The PAS cells were filled with granules as usual, and

![Fig. 6. Absorption spectrum of the extract of the thymus and pancreas from adult Db mice injected with dithizone. Curve I: extract of the thymus. Curve II: extract of the pancreas.](image)

![Fig. 7. Pancreatic islet of an adult Db mouse 3 hours after the administration of alloxan. Degenerative changes in the B-cells (dark) are recognized. Aldehyde-fuchsia, counterstained with Masson's trichrome method. ×580](image)
no changes were observed in the reactivity to Schiff's reagent, aldehyde-fuchsin, Sudan black B, pyronine and so on. The nuclei of the cells showed no tendency to pyknosis. Therefore, it was concluded that alloxan does not affect PAS cells in any way, and affect only pancreatic B-cells.

III. Discussion.

PAS cells are considered to correspond to those described as "Infiltrationszellen" in the thymus of rabbits during pregnancy by FULCI (1913) and during lactation by BOMPIANI (1914), as "lipid-laden foamy cells" in the physiologically involuted thymus of mice by LOEWENTHAL and SMITH (1952), as "lipophages" in the thymus of old mice by SPICER (1960), as "PAS positive and sudanophilic cells" in the thymus of mice following the administration of hydrocortisone by ITO and HOSHINO (1962), and as "phagocytic PAS positive reticulum cells (PAS cells)" by METCALF and ISHIDATE (1961). FULCI speculated the possibility that "Infiltrationszellen" might be concerned with internal secretion. The granules contained were regarded as a chromolipoid by LOEWENTHAL and SMITH, and as glycolipin by ITO and HOSHINO. Both of the reports concluded that the granules reflect altered metabolism in the thymus. As shown in Table 1, PAS cells are positive to the Schmorl test for lipofuscin. On the basis of this result it is suggested that PAS cells contain lipofuscins.

PAS cells showed affinity to aldehyde-fuchsin without any preoxidation, and the affinity became stronger after the oxidation with potassium permanganate. CASSELLA (1942) observed positive PAS reaction in chromolipoid; ceroid in the rat liver and lipofuscins in the adrenal of man, guinea pig and mouse. Moreover, HOLCZINGER (1956) observed positive reaction to aldehyde-fuchsin in unsaturated fat in the adrenal of man and rat. Accordingly, taking into account the positive reaction in the Schmorl test, the PAS reaction and the affinity to aldehyde-fuchsin of PAS cells could be originated in lipofuscins, though the histochemical basis of aldehyde-fuchsin itself is not yet clarified definitely.

In the electron microscope observations, HOSHINO (1963), CLARK (1963) and KLU (1965) reported that some reticulum cells in the thymus contained several kinds of granules or vacuoles. According to KLU some of these vacuoles often contain structures, which appear to be debris of phagocytized lymphocytes. In the cytoplasm of PAS cells, METCALF (1951) observed pyknotic nuclear material seemingly of lymphocytic origin. As described above these pyknotic nuclei were also observed in the PAS cells in the present observation.

CSABA (1965) observed that besides the involution of the thymus induced by the administration of cortisone and testosterone, large and medium sized thymocytes
took up PAS-positive substance, altered it into a metachromatic substance and they developed into mast cells. BURNET (1965) reported a massive change of thymocytes into mast cells in NZB mice in which mast cells were more numerous at all ages than in other strains. Besides, he observed intensively PAS positive cells in the midst of mast cells. There arises a question whether PAS cells are concerned with the mastocytegenesis. From the present results, mast cells could be always distinguished from PAS cells by using some dyes, and no gradational forms between PAS cells and mast cells were observed. Moreover, using alcian blue-safranine reaction, granules as seen in young mast cells were not recognized in the PAS cells.

Since OKAMOTO (1942) reported the histochemical method of detecting zinc in tissues by diphenylthiocarbazine and dithizone, some modifications of this method have been devised. As mentioned above, dithizone reacts with some other kinds of metals than zinc. STAMPFL (1959) described the absorption maximum of various compounds of dithizone with different kinds of metals (Fig. 5).

As illustrated in Fig. 5, zinc-dithizone compound shows its absorption maximum at 535 m\(\mu\) wave length. The extract of the thymus examined in the present work showed its absorption maximum which precisely corresponds to that of zinc-dithizone compound determined by STAMPFL, whereas the extract of the pancreas showed an unspecific curve, probably due to the presence of other metals, for example, cadmium, nickel and cobalt contained in the pancreas; it was at least indicated in the present study that cadmium is contained in the pancreas. Thus, in spite of the unexpected coincidence of the PAS cells of the thymus and the islet cells of the pancreas containing a considerable amount of zinc, the mode of presence of the metal in the both types of cell seems different.

According to STAMPFL, not all of tissue zinc reacts with injected dithizone, but only a part of it that is free or loosely bound with tissue proteins. Therefore it is suggested that PAS cells contain zinc, free or loosely bound with proteins, a fact which has, as far as is known, not been described by previous investigators. The physiological meaning of this zinc remains unknown.

The difference in nature of the thymus PAS cells and the islet B-cells seems to have been decisively demonstrated in the present study by means of administration of alloxan. It caused the well known changes in B-cells, whereas the PAS cells showed no degenerative signs. No evidence was obtained to support a possibility that the PAS cells play a role as an extrapancreatic insulin source as suggested by PAN-SKY and HOUSE (1965).

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IV. Summary.

In the thymus of Db and AKR mice, a series of experiments were carried out in an attempt to reveal some properties characteristic of "PAS cells". These PAS positive and sudanophilic reticular cells scattered mainly in the cortex of the thymus showed
the affinity to aldehyde-fuchsin and a positive reaction to Schmorl test, indicating the presence of lipofuscins in the cells. Mast cells, which occur numerously in the mouse thymus and show histochemical reactions which are partly found also in the PAS cells, could always be distinguished from the latter with Nile blue, alcian blue-safranine and toluidine blue stainings. No transitional forms between PAS cells and mast cells were observed.

With reference to the recent proposition of PANSKY and HOUSE (1965) that the "aldehyde-fuchsin positive reticulum cells", probably identical with our PAS cells, correspond to the B-cells of pancreatic islets, a comparative study of the both types of cell was carried out. By means of histochemical method of OKAMOTO, PAS cells were found to contain as much zinc as in pancreatic islet cells. Spectrophotometric examination revealed, however, that the metal contained in the former cells was represented simply by zinc, whereas that in the latter by zinc and some other metals such as cadmium. The administration of alloxan did not have any effect on PAS cells, whereas it caused definite degenerative changes in pancreatic B-cells.

**References.**